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Effects of dietary supplementation of garlic (*Allium sativum*) extract on the resistance of *Clarias gariepinus* against *Edwardsiella tarda* infection

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Abstract

The effects of dietary supplementation of garlic (*Allium sativum*) extract on the growth and disease resistance of African catfish, *Clarias gariepinus* was evaluated. Also *in-vitro* evaluation of susceptibility of fish-borne multidrug resistant (MDR) pathogenic bacteria to aqueous extract of garlic was done. Aqueous garlic extract exhibited inhibitory activity against MDR bacteria and the degree of inhibition increased significantly with increasing concentration of garlic extract. The garlic supplementation at the rate of 10g/ kg feed improved the food conversion ratio, specific growth rate and protein efficiency ratio of *C. gariepinus* significantly ($p<0.05$). On the other hand, it increased the disease resistance of *C. gariepinus* significantly when challenged with *Edwardsiella tarda* ($p>0.05$). No significant difference was observed between plasma protein and glucose levels of the control treatment and garlic supplemented treatment during the 56 days of the experiment. Additionally significant differences in plasma protein and glucose levels of control and garlic supplemented treatments in 2 days and/or 15 days post-challenge. The varying levels of plasma glucose and protein suggested that the stress posed by the bacterial infection persisted in fish even after 15 days of challenge. The results demonstrated that bacterial infection can negatively alter blood biochemical profile of *C. gariepinus*, and dietary supplementation of garlic extract would help improve the resistance of fish to *E. tarda* infection in culture condition.

Keywords: Catfish, Bacterial infection, Multidrug resistant bacteria, Garlic extract, Inhibitory activity, Plasma glucose

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Introduction

The potency of garlic, *Allium sativum*, a member of family Alliaceae, has been acknowledged for >5000 years and traditionally used for ages to treat a wide array of diseases. The health benefits of garlic include reduction of risk factors for cardiovascular diseases and cancer, stimulation of immune function, enhanced foreign compound detoxification, radio-protection, restoration of physical strength, resistance to various stresses and potential anti-aging effects (Amagase *et al*., 2001). The practice of complementary and alternative medicine is now on the increase in developing countries in response to World Health Organization directives culminating in several pre-clinical and clinical studies that have provided the scientific basis for the efficacy of many plants used in folk medicine to treat infections (Dilhuydy, 2003). Earlier reports documented antimicrobial effects of garlic against human pathogens such as *Helicobacter pylori* (Cellini *et al*., 1996), *Streptococcus pyrogenes, Bacillus cereus, Escherichia coli, Klebsiella pneumoniae* (Saravanan *et al*., 2010) and *Staphylococcus aureus* (Bjarnsholt *et al*., 2005) and fish pathogens like *Aeromonas hydrophila* (Chowdhury *et al*., 1991; Sahu *et al*., 2007), *Vibrio anguillarum*, *Streptococcus iniae* and *Edwardsiella tarda* (Wei and Musa, 2008; Kim *et al*., 2010). It should, however, be emphasised that inhibitory *in-vitro* activity does not necessarily reflect the *in-vivo* mode of action.

Garlic therapy can potentially fend off secondary infections, neutralize the chemicals used by the pathogen to destroy host tissue, mask host tissue, making it difficult for the pathogen to recognize it and deliver outright damage to the pathogen. Earlier studies documented that dietary supplementation of garlic enhance the intake, specific growth rate (SGR), food conversion ratio (FCR) and protein efficiency ratio (PER) in Nile tilapia, *Oreochromis niloticus* (Diab *et al*., 2002; Shalaby *et al*., 2006; Mesalhy *et al*., 2010; Ndong and Fall, 2011), shrimp, *Penaeus chinensis* (Aifang *et al*., 1997), goldfish, *Carassius auratus* (Sasmal *et al*., 2005) and rainbow trout, *Oncorhynchus mykiss* (Fazlolahzadeh *et al*., 2011; Nya and Austin, 2011). The continuous spread of multidrug resistant (MDR) bacterial pathogens has become a serious threat to fish and public health, and a major concern for infection control practitioners' worldwide (Sanders and Sanders, 1992). Use of antibiotics in aquaculture facilities may result in alterations in the microflora that could be unfavourable (Verner-Jeffreys *et al*., 2004). In lieu of the negative impacts of antibiotics on aquaculture, there is an urgent need on the prudent use of antibiotics in aquaculture facilities and control bacterial flora by other means. This communication reports *in-vitro* inhibitory effects of crude aqueous extract from garlic bulbs on MDR bacterial pathogens of fish as well as the effect of garlic supplemented feed on the growth performance and disease resistance of African catfish, *Clarias gariepinus*.

Materials and methods

Preparation of aqueous garlic extract

Fresh garlic, *A. sativum*, was brought from local market in Kolkata, India. The garlic was peeled and weighed. The bulbs were washed twice in distilled water. The washed garlic bulbs weighing 30 g were homogenized using a food processor with 60 mL of physiological saline (0.85 % NaCl). The homogenate was filtered through Advantec 125 mm filter paper and then centrifuged for 15 min at 6000 rpm in a refrigerated centrifuge at 20°C. The supernatant was then filter sterilized through a cellulose acetate membrane filter (Sartorius AG: pore size, 0.2 µm) under negative pressure. The sterile filtrate was taken as aqueous garlic extract and used immediately for *in-vitro* assays.

In-vitro inhibition of aqueous garlic extract by well diffusion assay

Five MDR bacterial pathogens, viz. *Aeromonas caviae, A. sobria, A. veronii, Edwardsiella tarda* and *Serratia fonticola* were isolated from diseased catfish, *C. gariepinus*, and maintained on tryptone soya agar (TSA) slants as collections of the Department of Aquatic Animal Health and used for assays (Table 1). TSA with 1.5 % agar and soft TSA (tryptone soya broth (TSB)+0.7% agar) were used as growth and overlay media for *in-vitro* assay, respectively. The anti-bacterial activity of aqueous garlic extract against the MDR bacteria was determined by well diffusion assay (Tagg and McGiven, 1971). The filter sterile aqueous garlic extract was added in to the wells of 5 mm diameter on TSA at 25 µL/well in duplicate. Control wells received only 25 µL sterile saline. The extract was allowed to diffuse in to the medium for 30 min. The plates were then overlaid with 10 mL soft TSA seeded with 10 µL of 20 h old respective bacterial culture so as to get a concentration of 10^6 cells and spread evenly to cover the entire area. The plates were incubated for 24h in an incubator at 30°C and the zone of inhibition was recorded in mm.

Table 1: Antibiotic sensitivity of catfish-borne bacterial species used in the *in-vitro* assay.

Bacterial species	**Antibiogram**					
	C, 30 µg	**F, 5 µg**	**G, 10 µg**	**N, 300 µg**	**O, 30 µg**	**T, 25 µg**
Aeromonas caviae	S	I	R	S	S	I
Aeromonas sobria	S	R	I	I	S	R
Aeromonas veronii	S	R	S	I	R	R
Edwardsiella tarda	S	R	S	S	R	R
Serratia fonticola	S	R	I	I	R	I

C: Chloramphenicol, F: Ciprofloxacin, G: Gentamicin, N: Nitrofurantoin, O: Oxytetracycline, T: Co-trimoxazole. R: Resistant, I: Intermediate, S: Sensitive.

Growth inhibition of MDR bacteria by aqueous garlic extract by broth dilution assay

The growth inhibition of MDR bacteria was determined by aqueous garlic extract following broth dilution assay (Kumar and Berwal, 1998) with slight modification. The tubes containing TSB were supplemented with different levels of sterile aqueous garlic extract at concentrations of 0, 0.5, 1.0, 1.5, 2.0 and 2.5%. The tubes were then inoculated

separately with MDR bacterial strains, viz., *A. caviae, A. sobria, A. veronii, E. tarda and S. fonticola* at a concentration of 10^6/mL separately. The initial optical density (OD) was noted at 620 nm using double beam UV vis spectrophotometer. The tubes were then incubated at 30ºC for 24 h and growth of the organisms was observed spectrophotometrically as turbidity (i.e., OD). The difference between the final (24h) and initial (0 h) OD values was interpreted as the growth of bacteria, whereas comparison of the differential readings (growth of bacteria) with the respective control readings depicted the inhibitory effect of garlic on the MDR bacterial pathogens. The growth inhibition of garlic was determined by plotting change in OD against the concentration of garlic extract.

Preparation of experimental feed

A commercial pellet feed (4mm size) containing crude protein 28%, crude fat 3%, crude fiber 5% and moisture 10% was used as a basal feed. Fresh garlic, *A. sativum* was peeled, washed thoroughly, weighed and the bulbs were ground to fine paste. The garlic paste was admixed with the basal feed at the rate of 10g/kg feed using banana as binder at 100g banana paste/kg feed (garlic supplemented feed). In control feed, binder alone (100g banana paste/kg basal feed) was added in to the basal feed. The garlic supplemented and control feeds were air dried for two days at room temperature (26-32°C) and stored in airtight plastic containers separately.

Experimental fish, growth condition and feeding experiment

The African catfish, *Clarias gariepinus*, (n=150) of size 10.16±1.05g were procured from Battala fish market, Naihati, West Bengal, India. The fish, on receipt, were disinfected by placing in 5-ppm potassium permanganate ($KMnO_4$) solution for up to 15min and transferred to circular FRP tanks of 500L capacity containing borewell water. The weak fish were removed immediately and the healthy ones were stocked at the rate of 75 fish/tank. The fish were fed with basal feed on demand twice daily. All the fish were maintained in such condition for at least 15 days prior to experimentation. The wastes and faecal matter were siphoned out every 3rd days. Prior to experimentation 14 healthy fish (11.06±1.25g) were transferred to six glass aquaria of size 60 cm L x 45cm B x 30cm H containing 35L water and acclimatized for 3 days. On the first day, two fish from each aquarium, one each for bacteriology and blood biochemistry, were removed and analyzed as described below. Thus, the effective numbers of fish used for the feeding trials were 12 each in respective aquaria.

During the experimental period of 56 days, the fish of treatment group in triplicate (G1, G2 and G3) were fed with garlic supplemented feed and those of control in triplicate (C1, C2 and C3) were fed with control feed at the rate 5 % of the body weight daily in 2 split doses. The wastes and faecal matter were siphoned out and water was exchanged on every 3rd day. The fish were observed for mortality daily. The length (cm) and weight (g) of the fish were noted on 0, 7, 14, 28, 42 and 56 days of culture and fish growth

parameters were estimated as described in Halver and Hardy (2002).

Mean weight increment (g)= Final average body weight – Initial average body weight

Mean length increment (mm)= Final average body length – Initial average body length

Total wet weight gain (g)= (Final wet weight at the end of the experiment + Wet weight of dead fish) – Initial wet weight

$$\text{Mean survival (\%)} = \frac{\text{Number of fish survived at the end of the experiment}}{\text{Number of fish stocked at the start of the experiment}} \times 100$$

$$\text{FCR} = \frac{\text{Total feed consumed on dry weight basis (g)}}{\text{Growth in terms of wet weight gain (g)}}$$

$$\text{SGR} = \frac{\ln W_t - \ln W_o}{\text{Days of culture}} \times 100$$

Where: W_t = Final average wet weight (g); W_o = Initial average wet weight (g)

$$\text{PER} = \frac{\text{Increase in body weight (g)}}{\text{Protein consumed (i.e. 28\% of feed)}}$$

Bacteriological analyses

The bacteria associated with the gut of *C. gariepinus* were enumerated as described below. In brief, one fish from each tank was scooped out and transferred to sterile container on day one and on day 56. The fish were killed by placing ice cubes in sterile containers, dissected-out and the entire gut removed aseptically. Care was taken to prevent contamination from entrails. The gut tissues along with contents of each group were aseptically transferred to pre-weighed sterile glass tubes containing 10mL physiological saline. The saline along with fish gut was weighed to get the weight of gut and its contents. The pooled gut and contents of each group were then macerated using a sterile glass rod and thoroughly mixed using a vortex mixer. The thoroughly macerated and vortexed gut samples were diluted by 10 fold serial dilution in saline to appropriate levels and used for the enumeration of different groups of bacteria immediately.

The bacterial groups enumerated following the standard methodology (APHA, 1992) include total viable counts (TVCs), motile aeromonads counts (MACs), lactose fermenters (Lac^+) and lactose non-fermenters (Lac^-), oxytetracycline (OTC) resistant bacterial counts (ORBCs) and total coliforms (TCs). Aliquots (0.1 mL each) of appropriately diluted gut samples were spread on to TSA for TVCs, starch ampicillin agar (SAA; Palumbo *et al*., 1985) for MACs, MacConkey agar (MCA) for Lac^+ and Lac^- bacteria and TSA supplemented with OTC at 25-µg/mL for ORBCs in duplicate. All plates were incubated at 30°C for 24-96h and the colonies were counted. The seeded SAA plates were flooded with iodine solution after 24h of incubation. The ampicillin resistant, amylase positive and yellow colonies were counted as

presumptive motile aeromonads. The most probable number (MPN) five tube technique was followed to enumerate total coliforms. Aliquots (1.0 mL) of appropriately diluted samples were inoculated into respective tubes containing 10 mL lactose broth and inverted Durham's tube. The tubes were incubated at 30 °C for 48 h and observed for gas formation. The number of positive tubes from each dilution was noted and referred to the McCardy table to get the MPN total coliforms count/g gut (APHA, 1992).

Resistance of C. gariepinus to E. tarda infection: Experimental fish and set up

On day 56, two fish from each tank were removed and used immediately for bacteriology and blood biochemistry. The remaining *C. gariepinus* of size 29.28±0.96 g were used for experimental *E. tarda* infection. For this experiment a total of 6 glass aquaria of size 60 cm L x 45 cm B x 30 cm H were cleaned first with 20 ppm chlorinated water (sodium hypochlorite), and washed thoroughly in tap water and dried for 3 days. All glass aquaria were filled with clean water to a volume of 25L each and conditioned for few days. The fish of garlic supplemented and control aquaria treatments were released in to the experimental aquaria at the rate of 10 fish/ aquarium. The aquaria were labeled as garlic supplemented (G1a, G2a and G3a) and control (C1a, C2a and C3a) groups. The fish of buffer stock were used as positive and negative controls in triplicate in a similar way. All aquaria were covered with nylon netting for adequate protection.

Preparation of bacterial cell suspension

A MDR strain of *E. tarda*, resistant to ciprofloxacin (5-µg/disc), oxytetracycline (30-µg/disc) and co-trimoxazole (25-µg/disc) and maintained on TSA slant, was streaked on to TSA plate and incubated at 30°C for 24h to get young culture. One young discrete colony of this strain on TSA was aseptically picked, transferred to 10mL TSB and incubated at 30°C for 24h. Mass culture was done in 300mL TSB at 30°C for 24h and centrifuged at 6000 rpm at 20°C for 15 min to harvest cells. The obtained pellets were washed thrice with physiological saline and finally suspended in 5 mL saline. The number of bacterial cells in suspension was determined by spread plating on TSA.

Bacterial challenge test

The garlic supplemented and control fed fish were injected intramuscularly with suitably diluted bacterial cell suspension containing *E. tarda* at the rate of 0.1 mL/fish at the base of dorsal fin, in such a way to get 8.60×10^7 cells/fish. Positive control fish received sterile saline at the rate of 0.1 mL/fish; while the negative control received no injection. One fish from each aquaria of garlic supplemented (G1a, G2a and G3a) and control (C1a, C2a and C3a) fed groups was removed on day 2 post-challenge for blood biochemistry analyses. The effective numbers of fish used for the challenge trials were 9 each in respective aquaria. The positive and negative control fish were not included in the assays. The test fish were maintained in their respective aquaria for 15 days and fed daily with basal feed on demand. Observation on mortality, external signs of

infections, cannibalism and behavioral changes were recorded daily.

Blood biochemical and statistical analyses

Blood samples were collected from one fish of each aquarium on day one and day 56 of feeding trials with garlic supplemented and control feeds, and 2nd day and 15th day post-challenge with *E. tarda* by tail ablation in to heparinized tubes. The blood cells were removed by centrifugation at 3000 rpm for 15 min and collected the plasma for biochemical analyses. Total plasma protein was estimated following Lowry *et al*. (1951). The plasma glucose was determined by glucose test kit (Autospan, Span Diagnostics Ltd, India) based on enzymatic GOD-POD method. ANOVA and Duncan's multiple range tests were used to determine the significant differences among treatments groups using SPSS program. The statistical significance of difference between the mean values was set as $p \leq 0.05$.

Results

The bacterial strains of the present study, isolated from diseased catfish, *C. gariepinus,* were sensitive to chloramphenicol and exhibited varying degrees of resistance to other antibiotics (Table 1). Aqueous garlic extract at 25µL level inhibited the MDR bacterial flora at varying degrees (Fig. 1). The well diffusion assay gave the largest inhibitory zone for *A. caviae* (18 mm) followed by *A. sobria* (13.50 mm), *A. veronii* (13.50 mm), *E. tarda* (9.5 mm) and *S. fonticola* (8 mm). The results of broth dilution assay are presented in Fig. 2. Significant differences existed in growth inhibition among the bacterial species ($p<0.05$). The inhibition pattern shown by *S. fonticola* and *E. tarda* was significantly different from that of *Aeromonas* spp. ($p<0.05$). The inhibition pattern of *S. fonticola* differed insignificantly to that of *E. tarda.* The differences among inhibition pattern of *Aeromonas* spp. were also insignificant ($p>0.05$). The garlic concentrations also had significant effect on growth inhibition of MDR bacteria ($p<0.05$). The rate of inhibition was more at 2.5 % level than that of other concentrations of garlic extract; while inhibition at 1.0 % and 0.5 % was insignificant ($p>0.05$).

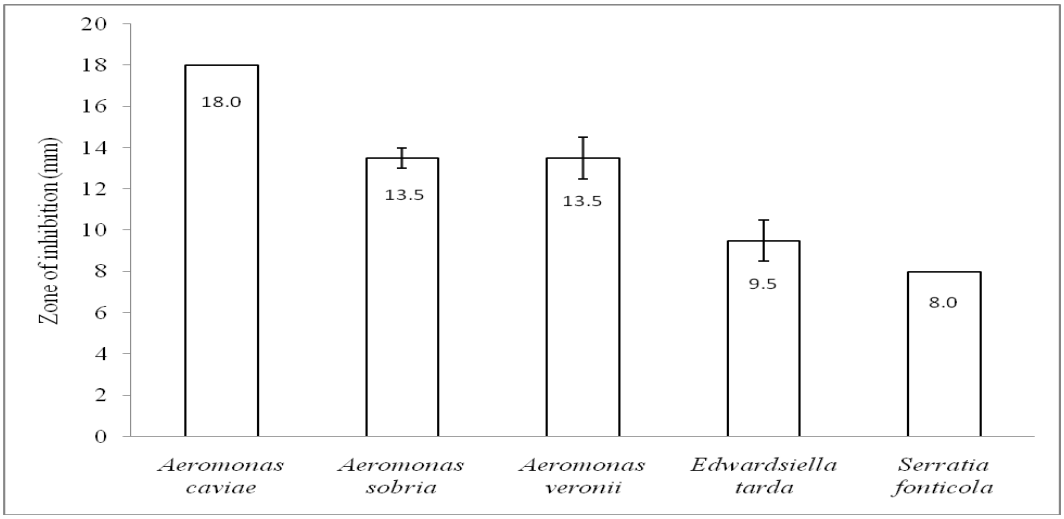

Figure 1: Inhibitory effect of aqueous garlic extract (25-µl) on multidrug resistant bacteria in well diffusion assay.

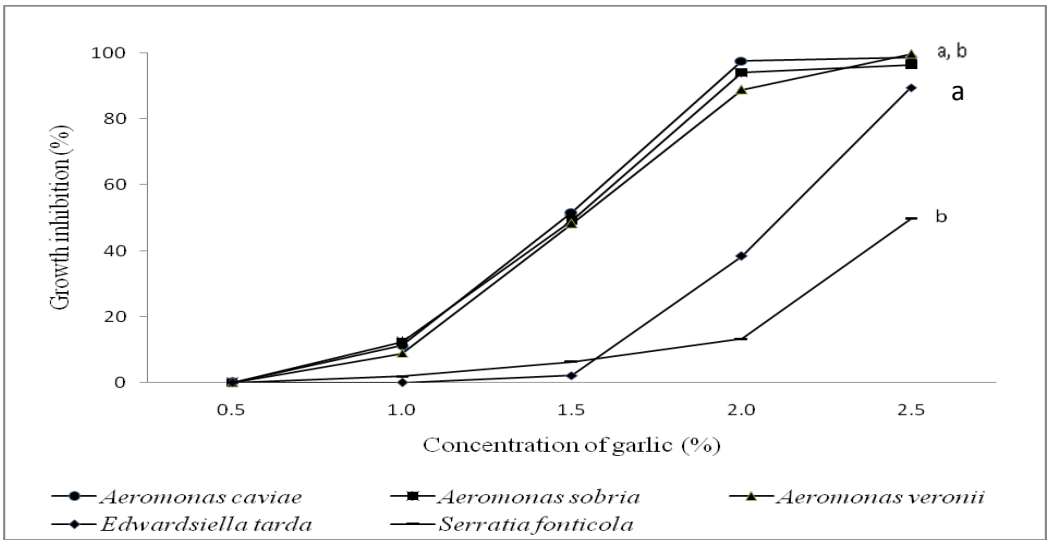

Figure 2: Growth inhibition of multidrug resistant bacteria by aqueous garlic extract at different concentrations in broth dilution assay. a-b: The line diagram sharing common superscripts differed significantly (p<0.05). The differences in growth inhibition among *Aeromonas* spp. were insignificant (p>0.05).

There existed significant differences in length and weight increment of *C. gariepinus* (Table 2) with increasing days of culture (p<0.05); while the difference between growth rate of garlic supplemented and control fed *C. gariepinus* was insignificant (p>0.05). Also the differences in growth parameters like FCR, SGR and PER (Table 3) of garlic supplemented fed fish and control treatments were found to be insignificant (p>0.05). The total viable counts in gut of all fish groups were always high, ranging from $7.95x10^8$/g to $2.05x10^9$/g. Motile aeromonads and lactose fermenters were the predominant bacteria in gut of *C. gariepinus*. The counts of lactose fermenters (Lac^+) and motile aeromonads in gut of garlic fed and control groups increased from the initial count; while

those of lactose non-fermenters (Lac^-) decreased markedly on day 56 in garlic fed fish. Further, the counts of oxytetracycline resistant bacteria ($2.13x10^6$–$1.45x10^7$/g) in gut of *C. gariepinus* increased from 0.27-0.48% of TVCs on day one to 0.56-0.71 % on day 56 (Table 4).

Table 2: Length and weight of *Clarias gariepinus* fed with garlic supplemented and control feeds.

Days of culture	Length (cm)		Weight (g)	
	Garlic fed Mean±SD*	Control Mean±SD*	Garlic fed Mean±SD*	Control Mean±SD*
0	10.82±0.18	10.78±0.10	10.53±0.71	11.60±1.80
7	11.43±0.35	11.50±0.22	13.97±2.57	13.10±0.70
14	11.77±0.28	12.07±0.42	15.13±2.42	16.27±0.21
28	13.07±0.42	13.37±0.46	17.87±3.46	19.57±1.32
42	14.43±0.68	14.52±0.64	28.13±6.33	28.37±4.11
56	15.42±0.94	15.35±0.79	29.60±4.80	29.67±3.21

*: On the basis of 12 observations.

Table 3: Growth parameters of *Clarias gariepinus* fed with garlic supplemented and control feeds.

Growth parameters	Garlic fed Mean ± SD	Control Mean ± SD
Mean weight increment (g)	18.42 ± 4.10	18.37 ± 1.46
Mean length increment (cm)	4.43 ± 0.90	4.57 ± 0.68
Total wet weight gain (g)	188.52 ± 21.02	183.67 ± 14.64
Mean survival (%)	100.00 ± 0.00	100.00 ± 0.00
Food conversion ratio	2.77 ± 0.16	2.91 ± 0.18
Specific growth rate	1.77 ± 0.19	1.70 ± 0.10
Protein efficiency ratio	1.26 ± 0.07	1.23 ± 0.08

Table 4: Counts of bacteria in the gut of *Clarias gariepinus* fed with garlic supplemented and control feeds.

Bacterial group	Days of culture and test group			
	1st day		56th day	
	Garlic fed	Control	Garlic fed	Control
Total viable counts, cfu/g	$8.80x10^8$	$7.95x10^8$	$1.10x10^9$	$2.05x10^9$
Motile aeromonads, cfu/g	$4.45x10^7$ (5.06)	$3.80x10^7$ (4.78)	$7.55x10^7$ (6.86)	$1.65x10^8$ (8.05)
Lactose non-fermenters (Lac^-), cfu/g	$6.15x10^6$ (0.70)	$4.25x10^6$ (0.53)	$2.43x10^5$ (0.02)	$6.05x10^6$ (0.30)
Lactose fermenters (Lac^+), cfu/g	$1.75x10^7$ (1.99)	$2.05x10^7$ (2.58)	$6.55x10^7$ (5.95)	$1.20x10^8$ (5.85)
Total coliforms, MPN/g	$2.80x10^7$ (3.18)	$3.00x10^7$ (3.77)	$1.10x10^7$ (1.00)	$5.00x10^7$ (2.44)
Oxytetracycline resistant bacteria, cfu/g	$4.25x10^6$ (0.48)	$2.13x10^6$ (0.27)	$6.15x10^6$ (0.56)	$1.45x10^7$ (0.71)

Values in parenthesis indicate proportion (%) of respective bacterial group in the total viable counts

The survival rate of garlic supplemented *C. gariepinus* treatment when challenged with *E. tarda* was significantly ($p<0.05$) higher (92.59%) than that of the control (74.08%) treatment fish (Table 5). The plasma protein and glucose levels of *C. gariepinus* when fed with garlic for 56 days and, 2nd day and 15th day post-

challenge with *E. tarda* are presented in Table 6. No significant differences (p>0.05) in the plasma protein and glucose levels of control and garlic supplemented fed *C. gariepinus* treatment on 56 days of experiment was observed. A significant reduction (p<0.05) in the plasma protein levels of 56th day control treatment and 2nd day post-challenge fish (p<0.05) was noted. Likewise, the increase in plasma glucose levels of 56th day control and/or garlic supplemented treatment with those of respective 2nd day post-challenge fish was also significant (p<0.05). The levels of plasma protein in 15th day post-challenge fish were observed to be significantly higher (p<0.05) than those of the 2nd day post-challenge fish and significantly lower than the 56th day garlic supplemented feed fed fish. Likewise, the levels of plasma glucose in 15th day post-challenge fish were observed to be significantly lower (p<0.05) than those of the 2nd day post-challenge fish and significantly higher than those of the 56th day garlic supplemented treatment fish. Similar results were observed in control treatment fish.

Table 5: Survival rate in challenged *Clarias gariepinus* fed with garlic supplemented and control feeds.

Group	Survival rate* (%)
Garlic fed	$92.59^{a} \pm 6.41$
Control	$74.08^{a} \pm 6.41$

*: In 15 days when challenged with *Edwardsiella tarda* at 8.60×10^{7} cells/fish.
a:Values showing common superscripts differed significantly (p<0.05)

Table 6: Changes in plasma protein and glucose levels of *Clarias gariepinus* when fed with garlic and, 2nd and 15th day post-challenge with *Edwardsiella tarda*.

Days of culture / post challenge	Treatment groups	Total plasma protein (g/100mL)	Plasma glucose (mg/dL)
1	Control	7.36 ± 0.29	88.66 ± 11.24
	Garlic fed	7.27 ± 0.22	89.11 ± 3.86
56	Control	7.13 ± 0.73^{a}	87.67 ± 9.07^{ad}
	Garlic fed	7.48 ± 0.51^{bc}	79.67 ± 3.51^{be}
2nd day post-challenge	Control	4.67 ± 0.49^{ad}	166.33 ± 11.24^{acf}
	Garlic fed	5.28 ± 0.41^{b}	140.66 ± 7.76^{bcg}
15th day post-challenge	Control	6.26 ± 0.14^{d}	108.33 ± 7.09^{df}
	Garlic fed	6.78 ± 0.28^{c}	101.67 ± 4.51^{eg}

a-g: Values sharing common superscripts within column differed significantly (p<0.05)

Discussion

The pathogenic bacteria of *C. gariepinus* were resistant to ciprofloxacin, oxytetracycline and co-trimoxazole, possibly because of their abuse in catfish aquaculture as noted earlier (Abraham and Bharathkumar, 2009). However, the aqueous garlic extract (25µL) inhibited these MDR bacteria at varying degrees. *Aeromonas* spp. were the most sensitive among the bacterial strains tested. The inhibition pattern shown by *S. fonticola* and *E. tarda* was significantly different from those of *Aeromonas* spp. The rate of bacterial inhibition was more at higher concentration of garlic extract. Likewise, Wei and Musa (2008) demonstrated that garlic extract was more effective to control

gram negative bacteria (except for *Citrobacter freundii* and *Escherichia coli*) than *Streptococcus agalactiae* and *Staphylococcus aureus.* Chowdhury *et al*. (1991) reported that extract obtained from garlic was highly effective against *A. hydrophila* and *Pseudomonas fluorescens* (MIC 0.6 mg/mL). Evidences support that the anti-microbial activity of garlic against bacterial pathogens can be through blocking the quorum sensing phenomenon as garlic reportedly blocks the quorum sensing (Bjarnsholt *et al*., 2005*;* Harjai *et al*., 2010). The results corroborate the observations of Saleem and Al-Delaimy (1982), who reported bacterial growth inhibition of *Bacillus cereus* ranging from 43.24 to 100 % by garlic extract, except for *Plesiomonas shigelloides* and *Staphylococcus* isolates.

The present study recorded insignificant differences between the growth parameters like FCR, SGR and PER of catfish fed with garlic supplemented and control feed ($p>0.05$). In contrast, several earlier studies reported that dietary garlic supplementation led to high growth performance in terms of enhanced SGR, FCR and PER in other fish species such as *O. niloticus* (Diab *et al*., 2002; Shalaby *et al*., 2006; Mesalhy *et al*., 2010), *C. auratus* (Sasmal *et al*., 2005), *O. mykiss* (Fazlolahzadeh *et al*., 2011; Nya and Austin, 2011). Garlic as dietary supplement and oral immunostimulant has been reported to enhance disease resistance, improve overall fish health and impact on growth and body composition specifically (Halver and Hardy, 2002). According to them, the feeding rates and fish size affect specific weight gain and feed conversion.

Most microbes are transients in aquatic animals and may change rapidly with the intrusion of microbes coming from water and food. As seen in Table 4, the TVCs in gut of fish were always high, ranging from $7.95x10^8$/g to $2.05x10^9$/g. The results are in conformity with Sasmal *et al*. (2005), who reported $>10^8$ cells/g fish gut when fed with garlic supplemented feed. The counts of lactose fermenters and motile aeromonads were unaffected in the gut of garlic supplemented and control fed fish, which increased with days of culture. The garlic feeding was particularly effective against the lactose non-fermenters as their counts decreased markedly on day 56 in garlic fed fish compared to control. Earlier studies (Sugita *et al*., 1992; Ringø *et al*., 1995) reported that the predominant species of bacteria found in the intestines of freshwater fish are *Aeromonas, Enterobacter, Flavobacterium, Pseudomonas* and *Acinetobacter*. In the present study, none of the bacterial groups of gut between the garlic fed and control fish differed much, probably due to an acidic environment in the gastric cavity, which can irreversibly neutralize alliinase (Amagase *et al*., 2001). These results indicated that garlic extract did not have any significant *in-vivo* inhibitory effect on fish gut bacterial flora when administered through feed. The results, however, contradict with those of *in-vitro* study (Figs. 1, 2), which demonstrated that garlic extract was capable of inhibiting *Aeromonas* spp. and *E. tarda*. Another probable reason is that the length of alimentary tract of *C. gariepinus* is much

shorter than that of other fish. Consequently, the time that garlic supplemented feed remains in alimentary tract of *C. gariepinus* is much shorter. This explains that garlic remains in alimentary tract of *C. gariepinus* for a short period, which is insufficient to allow it to interact with constantly changing intestinal bacterial flora, which fluctuates strongly on a daily basis. It was further observed that the counts of oxytetracycline resistant bacteria in the gut of *C. gariepinus* increased from 0.27- 0.48% of TVCs on day one to 0.56-0.71% on day 56, possibly due to the influence of pellet feed on ORBCs as suggested by McPhearson *et al*. (1991). The levels of ORBCs recorded in this study were similar to those of catfish *Pangasius pangasius* larvae (Abraham and Bharathkumar, 2009).

In the present study it was observed that the garlic supplemented *C. gariepinus* resisted bacterial infection. The survival rate of garlic fed *C. gariepinus* was significantly higher (92.59%) than that of the control treatment (74.08%), when challenged with *E. tarda*. The results of this study supported the previous findings of Sahu *et al*. (2007), who noted the effectiveness of 5 g and 10 g garlic/kg feed in rohu, *Labeo rohita* when challenged with *A. hydrophila.* Nya and Austin (2011) recorded 1g garlic/100 g feed as the most effective dose in terms of protecting rainbow trout, *O. mykiss* against challenge by *A. hydrophila*. In another study by Sasmal *et al*. (2005), the feed containing 1g garlic/100g significantly improved the food conversion ratio and disease resistance of *C. auratus* to *P. fluorescens* challenge. The observations of Cho and Lee (2012) also indicated that onion (*Allium cepa* L.) powder can be an effective immunostimulant to lower mortality of olive flounder *Paralichthys olivaceus* infected with *E. tarda*.

Plasma glucose is a sensitive reliable indicator of stress in fish. The results of varying levels of plasma glucose and protein suggested that the stress posed by bacterial infection persisted in fish even after 15 days of challenge. The increment in plasma glucose levels of challenged garlic supplemented treatment fish were significantly lower (p<0.05) than those of the challenged control fish. This probably suggested induced hyperglycaemia resulting from the incomplete metabolism of blood sugar and impaired osmoregulation due to *E. tarda* challenge. The observations of this study on the insignificant effect of dietary supplementation of garlic on weight gain and feed efficiency, but favourable effect on reducing the mortality of *C. gariepinus* after *E. tarda* infection indicated that garlic could be useful as an immunostimulant rather than growth-enhancing additive. The fact is that blood biochemical values are not commonly used as a diagnostic tool in fish medicine, partly because of the lack of reference intervals for various fish species, and also because changes in blood analysis associated with specific diseases and metabolic disorders are not well characterized with sufficient background data. Findings of this study, however, demonstrated that the bacterial infection can negatively alter the blood biochemical profile of *C. gariepinus*, and dietary supplementation of garlic (*A. sativum*) extract would help improve the

resistance of fish to bacterial infection and mitigating the mortality. Dietary supplementation of garlic can, therefore, be a possibility of obtaining a cheaper and potent alternative way of preventing *E. tarda* septicemia in catfish aquaculture. Further research on the supplementation of garlic or constituents of garlic in fish feeds and understanding their immunostimulatory action could contribute to more environmental friendly catfish production.

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